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Diversity of *Azolla japonica* in Japan, Analyzed by Random Amplified Polymorphic DNA

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To understand biological nature of *Azolla japonica*, whose taxonomy is controversial among researchers, similarities of twenty seven field populations of *A. japonica* that were collected from various areas in Japan were examined by random amplified polymorphic DNA (RAPD) method, in reference to other *Azolla* species. The present study revealed the presence of at least two distinct groups in *A. japonica*; one that includes *A. filiculoides* specimen is distributed in a limited area, and another is distributed in wider areas.

Key words: *Azolla*, *A. japonica, A. filiculoides, A. rubra*, DNA

There are two apparently indigenous *Azolla* species in Japan, *A. imbricata* (Roxb. ex Griff.) Nakai and *A. japonica* Fr. et Sav.(Iwatsuki 1992). *A. imbricata* is now considered synonymous with *A. pinnata*, and is placed in subsp. *asiatica* (Saunders and Fowler 1992, 1993). Taxonomic status of *A. japonica* is, however, controversial among researchers. Some papers have described that *A. japonica* closely resembles *A. filiculoides* (Abe and Yoshii 1913, Shimura 1966, Tanaka 1994). On the other hand, Lumpkin and Plucknett (1980) regarded it as *A. rubra* that is usually considered as a variety or subspecies of *A. filiculoides*. Using *Azolla* Germplasm Collection in the International Rice Research Institute (IRRI) (Watanabe et al. 1992, Watanabe and Hove 1996), Dunham (1986) certified that on the basis of an leaf trichome *A. japonica* was synonymous with *A. filiculoides*, and other researchers adopted the same view (Zimmerman et al. 1989, 1991a, 1991b Stergianou and Fowler 1990). However, their studies were based on only three specimens of *A. japonica* deposited at IRRI.

By randomly amplified polymorphic DNA (RAPD) method, Coppenolle et al. (1993) analyzed phylogenetic relationships in *Azolla* species and showed a dendrogram summarizing relationships among 25 accessions of *Azolla*. *Azolla* was grouped to three groups, species belonging to section *Azolla*, *A. pinnata*, and *A.nilotica*. Among species belonging to section *Azolla*, *A.filiculoides* was clearly distinguished from the rest of species. Three species belonging to section *Azolla*, *A. mexicana*, *A. caroliniana*, and *A.microphylla* were not distinctly separated. This finding was well coincide with that based on zymogram and restriction fragment length polymorphism (RFLP) analyses by

Zimmerman et al. (1991a, 1991b).

The present study was undertaken to clarify the genetic background for the taxonomy of *A. japonica* collected from field populations in Japan. We analyzed them by RAPD method and revealed the presence of at least two distinct groups.

Materials and Methods

Collection of Azolla japonica

Samples in *A. japonica* of different populations were collected from southern part of Japan, Chiba to Fukuoka, and deposited in the Museum of Nature and Human Activities, Hyogo and Faculty of Bioresources, Mie University (Table 1). Reference species were obtained from IRRI and listed with their IRRI code numbers in Table 1 (Watanabe et al. 1992). *Azolla* plants were grown on nitrogen free IRRI *Azolla* medium (Watanabe et al. 1992) in greenhouse during summer season or in growth chamber (12 hr-24°C-light/12 hr-18°C-dark cycle with 4000–6000 lx light).

DNA extraction

Before harvesting of plants, they were placed under a dark condition for more than 12 hr to decrease any accumulated carbohydrates that may interfere with DNA extraction. From the plants harvested, the leaves were sampled following removing of the roots and washed first with running tap water for more than 10 min and then with distilled water. The washed and blotted dry leaves were homogenized to a fine powder with a mortar and pestle cooled with liquid nitrogen. DNA was extracted from the leaf powder as described by Rogers and Bendich (1988). The leaf DNA was purified on CsCl gradient after centrifugation overnight at 100,000 rpm. The DNA thus obtained was a mixture of DNAs from both the *Azolla* plants and symbiotic cyanobacteria, *Anabaena azollae*.

PCR amplification and agarose gel electrophoresis

PCR reaction mixture was prepared with 2.5 µl buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 2.0 µl dNTP mixture (2.5 mM each), 0.2 µl Taq DNA polymerase (5 units/µl, Takara, Japan), 3.3 µl primer (1.5 mM), 10 µl DNA (40 ng), and distilled water to make a total volume of 25 µl. One drop of light mineral oil was added to prevent evaporation. After heating at 92 °C for 3 min, PCR amplification were performed with 45 cycles of 92 °C for 1 min, 35 °C for 2 min, and 72 °C for 3 min in DNA Thermal Cycler (Takara, Japan), followed by extension at 72 °C for 7 min. According to the experimental results obtained by Coppenolle et al. (1993), the following seven primers were synthesized (Sawady Technology, Japan); B03: `CATCCCCCTG`, B08: `GTCCACACGG`, B13: `TTCCCCCGCT`, C08: `TGGACCGTCC`, C09: `CTCACCGCC`, C19: `GTTGCCAGCC` and M10: `TCTGGCGCAC`. The PCR products were separated by electrophoresis on a 2 % agarose gel containing ethidium bromide (1 µg/ml) at 100 V for ca. 1 hr. Photographs of the gels were taken under UV light. Genetic distance (dissimilarity index) was based on the proportion of unshared DNA bands in pairwise comparisons between accessions. Dendrogram was constructed by a centroid method for hierarchial clustering from matrix of the genetic distances (SAS Institute, USA).

Results and Discussion

In the first set of experiment, *Azolla* accessions No. 1,2,3,5,6,7,8,9,10,11, and 28 were analyzed together with reference accessions from IRRI collection; FI1005, FI1035, MI4018, MI4030 and RU6503. A lot of DNA bands were detected from PCR products using seven primers in these accessions. DNA band at the same migration distance in the gel was scored as a single RAPD marker for each primer. From seven to 22 RAPD markers were noted for each primer. Clear RAPD patterns such as the ones shown in Fig. 1 (PCR fragments amplified with a primer C09 and B03) were obtained. Among *A. japonica* accessions (lane 5–12), bands patterns of lanes 5–8 are clearly distinguished from those of lanes 9-12. Band patterns of FI1005 (lane 1) and FI1035 (lane 2) were more close to those of lanes 5–8 than those of lanes 8–12.

The accession of MI4030 (lane 4) is a hybrid between *A. microphylla* (lane 3) and *A. filiculoides* (lane 1) as reported by Do et al.(1989), and exhibited both of the DNA bands from the male and female parent species.

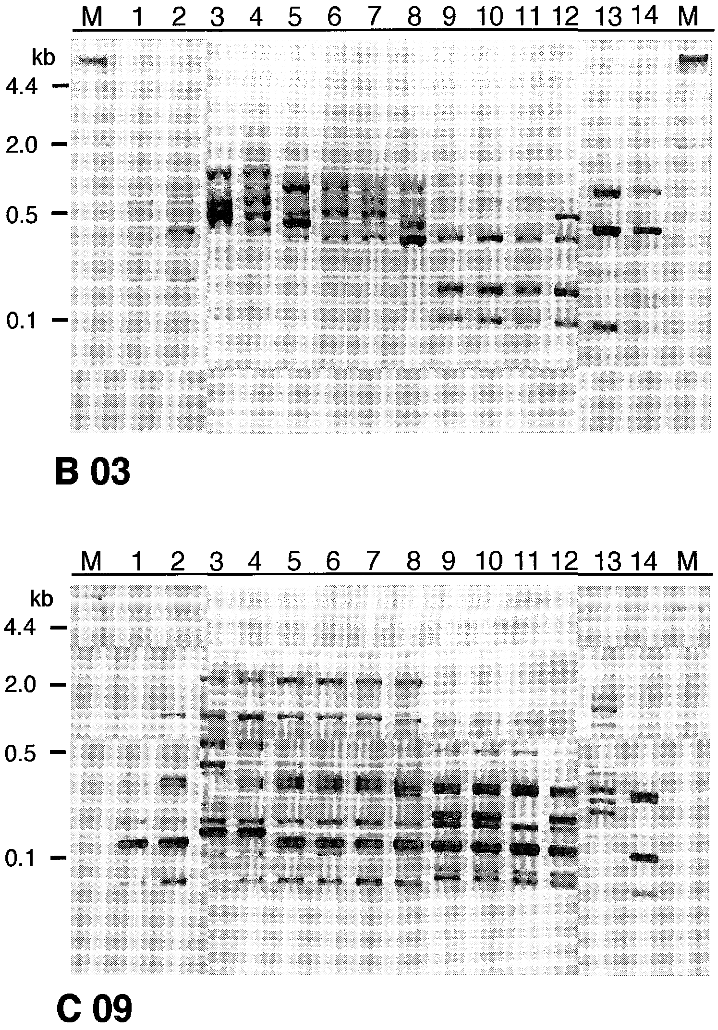
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Fig. 1. DNA polymorphism of 14 *Azolla* accessions obtained by amplification of total DNA from *Azolla-Anabaena* symbionts using a random primer B03 (upper) and C09 (lower). The lane numbers correspond to: 1; FI1005, 2; FI1035, 3; MI4018, 4; MI4030, 5; Nara-1, 6; Osaka, 7; Kyoto, 8; Chikugo, 9; Nasa, 10, Oharu, 11, Noma, 12, Momotori-2, 13, Okuda-2 (*A. pinnata*),14; RU6503, M; DNA size markers.

Table 1. List of Azolla accessions used

Azolla from Japan

No.	Code	Site	Collector	Collected year	Group
1	Osaka	Settsu-shi, Osaka Pref.	Shiomi	1993	A
2	Kyoto	Kyotanabe-shi, Kyoto Pref.	Shiomi	?	A
3	Nara-1	Nara-Park, Nara-shi, Nara Pref.	Shiomi	1993	A
4	Nara-2	Nara-Park, Nara-shi, Nara Pref.	Watanabe	1995	A
5	Chikugo	Ooki-machi, Miduma-gun, Fukuoka Pref.	Shogomori	1993	A
6	Noma	Noma, Mihama-cho, Chita-gun, Aichi Pref.	Watanabe	1994	B
7	Okuda-1	Okuda, Mihama-cho, Chita-gun, Aichi Pref.	Watanabe	1994	B
8	Momotori-1	Momotori, Momotori-cho., Toba-shi, Mie Pref.	Watanabe	1994	B
9	Momotori-2	Momotori, Momotori-cho., Toba-shi, Mie Pref.	Watanabe	1994	B
10	Oharu	Oharu, Momotori-cho., Toba-shi, Mie Pref.	Watanabe	1994	B
11	Nasa	Nasa, Momotori-cho., Toba-shi, Mie Pref.	Watanabe	1994	B
12	Futami	Matsushita, Doai-gun, Futami-cho, Mie Pref.	Watanabe	1994	B
13	Chiba-5	Takatsukayama, Chikura-machi, Awa-gun, Chiba Pref.	Yamada	1995	B
14	Chiba-1	Higashi-Namimi, Chosei-gun, Ichinomiya-cho, Chiba Pref.	Nakamura	1995	B
15	Chiba-2	Kami-Izumi, Sodegaura-shi, Chiba Pref.	Kawana	1995	B
16	Chiba-3	Masuma, Negami, Miyoshi-mura, Awa-gun, Chiba Pref.	Suzuki	1995	B
17	Chiba-4	Komoguchi, Tateyama-shi, Chiba Pref.	Suzuki	1995	B
18	Ishikawa-1	Bessho, Nakashima-cho, Kashima-gun, Ishiwawa Pref.	Suzuki & Kawahara	1995	B
19	Ishikawa-2	Hanami, Noto-cho, Fugeshi-gun, Ishikawa Pref.	Suzuki	1995	B
20	Nara-3	Nijou-Minami, Nara-shi, Nara Pref.	Suzuki	1995	A
21	Osaka-5	Shukubou-chou, Higashi Osaka-shi, Osaka Pref.	Suzuki & Seto	1995	A
22	Osaka-7	Ooizumi Ryokuchi, Sakai-shi, Osaka Pref.	Suzuki	1995	A
23	Hyogo-1	Tai, Toyooka-shi, Hyogo Pref.	Suzuki	1995	B
24	Okayama-1	Omati, Okayama-shi, Okayama Pref.	Suzuki & Kobatake	1995	B
25	Shimane-1	Oashi, Shimane-cho, Yatsuka-gun, Shimane Pref.	Sugimura	1995	B
26	Ehime-1	Nishi-Teizui, Saijou-shi, Ehime Pref.	Suzuki & Fujita	1995	B
27	Matsubara	Matsubara-shi, Osaka Pref.	Shiomi	1995	B
28	Okuda-2	Okuda, Mihama-cho, Chita-gun, Aichi Pref.	Watanabe	1994	*A.pinnata*

Reference accessions

Code	Species	Characteristics	Collector	Year
FI1005	*A. filiculoides*	Hanburg, Germany	Scharpenseel	1980
FI1035	*A. filiculoides*	*Anabaena* from *A.microphylla*1)	Lin Cang	1986
MI4018	*A. microphylla*	Paraguay	Rains	1981
MI4510	*A. microphylla*	Philippines2)	Hove Van	1986
MI4030	*A. microphylla*	Hybrid with *A. fliculoides*3)	Do V. C.	1985
RU6503	*A. rubra*	New Zealand	Hove Van	1986

1)Symbiotic *Anabaena* was from *A. microphylla.* Method of obtaining this accession was described by Lin et al. (1989).

2)MI4018 was introduced to Mindanao, Philippines in 1980, and re-collected from the introduced site.

3)Method of getting this accession was described by Do et al. (1989).

To quantify mutual relationships of the accessions examined, dendrogram from distance matrix was built as shown in Fig. 2.

As reported by Coppenolle et al.(1993), the accession of *A. pinnata* were found to be most distantly related to all the accessions examined.

Azolla japonica accessions were clustered in at least two groups, as indicated in the boxes in Fig. 2. These two groups are hereafter designated Groups A and B. The accessions of Nara-1, Osaka, Kyoto and Chikugo were included into Group A, which included two accessions of *A. filiculoides*, FI1005 and FI1035. The accessions of Nasa, Oharu, Noma, and Momotori-2 were included into Group B.

To elucidate the presence of two groups in *A. japonica* revealed from a limited number of field populations as mentioned above, *A. japonica* populations were collected from much wider areas in Japan. In the second set of experiment, twenty seven accessions including reference accessions from IRRI (FI1005, FI1035, MI4510 and RU6503) were used for RAPD analysis with seven primers. Distinct amplification patterns such as one shown in Fig. 3 (C08) were obtained. From amplification patterns with seven primers, twenty six RAPD markers were identified. Based on these markers, the genetic distances between two accessions of *Azolla* were calculated by pairwise comparisons. Figure 4 shows a dendrogram drawn from the distance matrix. The dendrogram clearly shows that *A, japonica* accessions were clustered at least two groups, as observed in the first experiment. The accessions used in the first set of experiment were grouped in the same group as that observed in the second experiment, although relative topography within each cluster differed between the two sets of experiments (Figs. 2, 4). From Fig. 4, it is seen that the accessions included in Group A were Osaka, Matsubara, Osaka-5, Osaka-7, Nara-3 and Chikugo which are distributed in limited areas around Osaka to Nara and in Fukuoka. Accessions of *A.filiculoides* (FI1005, FI1035) were included in the Group A. The Group B includes the accessions collected from wider areas in Japan, that is, Oharu, Futami, Noma, Ishikawa-2, Chiba-4, Ehime-1, Shimane-1, Chiba-2, Okuda-1, Chiba-5,

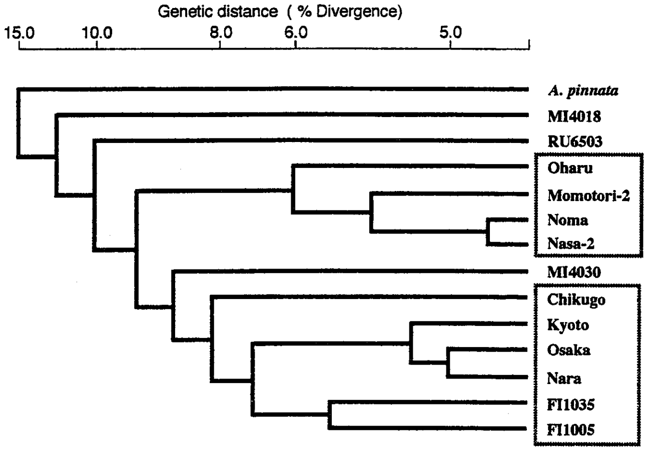

Fig. 2. Dendrogram demonstrating relationships among *Azolla* accessions shown in Fig. 1. Two groups are indicated in the boxes . The genetic distance (% divergence) is shown above the dendrogram.

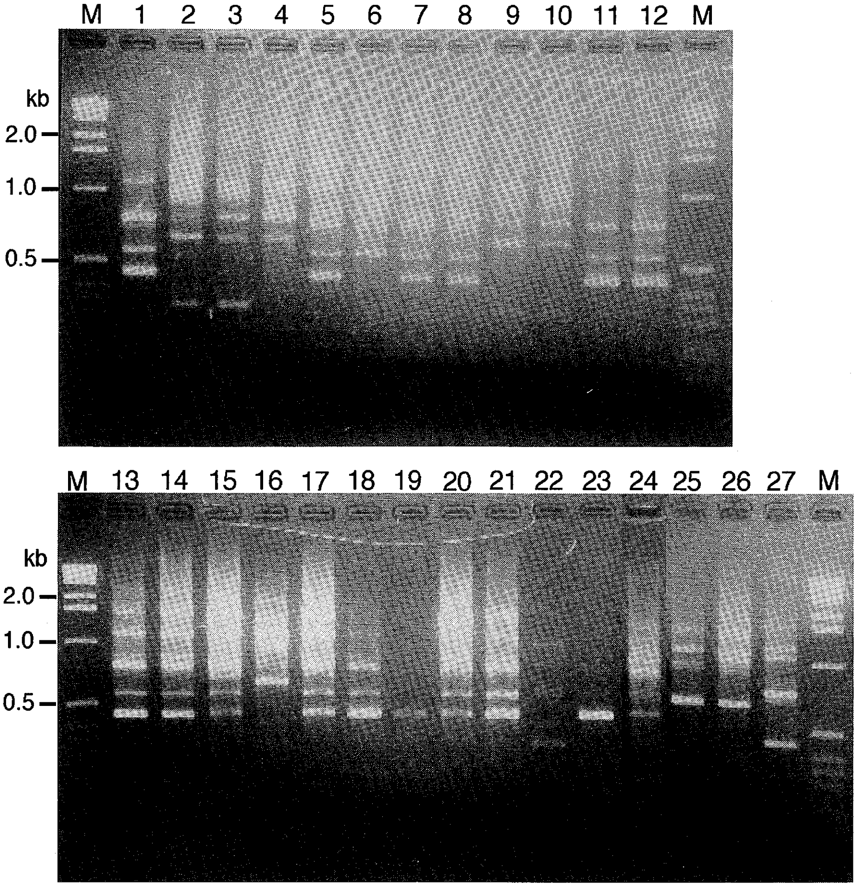
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Fig. 3. DNA polymorphism of 27 *Azolla* accessions obtained by amplification of total DNA from *Azolla-Anabaena* symbionts using a random primer C08. The lane numbers correspond to: 1; Oharu, 2; Osaka, 3; Nara-1, 4; Chikugo, 5; Futami, 6; MI4510, 7; Noma, 8; Chiba-3, 9; Oska-7,10; Osaka-5, 11; Ishikawa-2, 12; Ishikawa-1, 13; Chiba-2, 14; Chiba-4, 15; Ehime-1, 16; Nara-3, 17; Shimane-1, 18; Okayama-1, 19; Hyogo-1, 20; Okuda-1, 21; Chiba-5, 22; Nara-2, 23; Chiba-1, 24; Matsubara, 25; FI1001, 26; FI1035, 27; RU6503, M; DNA size markers.

Chiba-3, Ishikawa-1, Okayama-1 and Hyogo-1. *Azolla rubra* (RU6503) from New Zealand was not clustered into this group. Both the Groups A and B were also distinguished from *A. microphylla* (MI4510). Thus, the presence of at least two groups of *A. japonica* was confirmed using many populations from wider areas in Japan. This grouping is shown in the last column in Table 1.

Accessions of Nara-1, Nara-2 and Chiba-1 are not included in the dendrogram, shown in Fig. 4 because of incomplete band separation of PCR products using some primers. Accessions Nara-1 and Nara-2 should be included into Group A, and Chiba-1, Group B due to the presence of PCR products unique to each group (data not shown).

Suzuki, one of the authors, also identified the presence of at least two groups in the

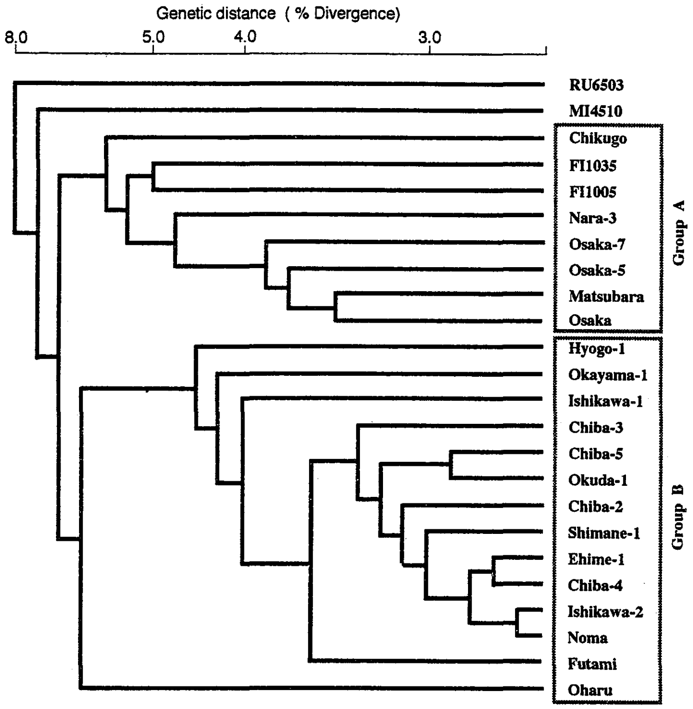

Fig. 4. Dendrogram demonstrating relationships among *Azolla* accessions. Two groups are indicated in the boxes. The genetic distance (% divergence) is shown above the dendrogram.

populations of *A. japonica* by isozyme analysis using almost the same populations as those used in the present RAPD analysis (unpublished). Grouping by isozymes exactly matched that by RAPD as reported here. The IRRI accessions of *A. filiculoides* (FI1005, FI1035) were clustered into Group A.

The IRRI accessions from Japan (FI1603 from Osaka, FI1607 from Chikugo, FI1608 from Matsue, previously coded as RU6003, RU6007, RU6008, respectively) were identified as *A. filiculoides* by isozyme and leaf trichome morphology (Zimmerman et al. 1991a) and by DNA fingerprinting (Zimmerman et al. 1991b). These three accessions were also used by Dunham and Fowler (1987) and Stergianou and Fowler (1990) as *A. filiculoides*. These earlier studies also give the evidence that *A. filiculoides* was distributed in Japan. The accession FI1608 was collected from Matsue, but the population from Shimane in the current study was not of group A, including *A. filiculoides*. This discrepancy needs further examination using IRRI accessions collected from Japan, and other populations around Matsue.

None of accessions belonging to Group B was examined by previous researchers. The phylogeny and taxonomic treatment of Group B requires further chemotaxonomical, morphological and genetic studies

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References

Abe Y. and Yoshii K. 1913. Notes on the Japanese species of *Azolla*. Bot. Mag. Tokyo **27**: 379–381 (in Japanese).

Coppenolle Van B., Watanabe I., Hove Van C., Second G., Huang N. and MacCouch S.R. 1993. Genetic diversity and phylogeny analysis of *Azolla* based on DNA amplification by arbitrary primers. J. Genome **36**: 686–693.

Do V.C., Watanabe I., Zimmerman W.J., Lumpkin T.A. and Baillonville de Waha T. 1989. Sexual hybridization among *Azolla* species. Can. J. Bot. **67**: 3482–3485.

Dunham D.G. 1986. Taxonomic re-evaluation and species recognition of *Azolla* Lam with particular reference to section *Azolla*. Univ. of Portmouth, U.K. (Ph. D. thesis).

—— and Fowler K. 1987. Taxonomy and species recognition in *Azolla* Lam. *In*: *Azolla* Utilization, International Rice Research Institute, Los Baños, pp.7–16.

Iwatsuki K. 1992. Ferns and Fern Allies of Japan. pp. 311, Heibonsha, Tokyo, Japan (in Japanese).

Lin C., Liu Z.–Z., Zheng D.–Y., Tang L.–F. and Watanabe I. 1989. Re-establishment of symbiosis to *Anabaena*–free *Azolla*. Sci. China, Series B, **32**: 551–559.

Lumpkin T.A. and Plucknett D.A. 1980. *Azolla* as a green manure: use and management in crop production, Westview Press, Boulder.

Rogers S.O. and Bendich A.J. 1988. Extraction of DNA from plant tissues. *In*: S.A.Gelvin and R.A. Schilperoot, (eds.), Plant Molecular Biology Manual, Kluwer Academic Publishers, Dordrecht, pp. A6/1–11.

Saunders R.M.K. and Fowler K. 1992. A morphological taxonomic reversion of *Azolla* Lam. section *Rhizosperma* (Mey.) Mett.(Azollaceae). Bot. J. Lin. Soc. **109**: 329–357.

—— and Fowler K.1993. The supraspecific taxonomy and evolution of the fern genus *Azolla* (Azollaceae). Plant Syst. Evol. **184**: 175–193.

Shimura Y. 1966. General consideration of the different characters between *Azolla japonica* Fr. et Sav. and *Azolla* imbricata (Roxb.) Nakai from the morphological point of view. Bull. Coll. Edu., Natural Science, Shizuoka Univ. **17**: 40–45 (in Japanese).

Stergianou K.K. and Fowler K. 1990. Chromosome numbers and taxonomic implications in the fern genus *Azolla* (Azollaceae). Plant Syst. Evol. **173**: 223–239.

Tanaka T. 1994. Observation of *Azolla japonica*. Iden **498**: 108–111 (in Japanese).

Watanabe I., Roger P.A., Ladha J.K. and Hove Van C. 1992. Biofertilizer Germplasm Collections at IRRI, The International Rice Research Institute, Los Baños.

—— and Hove Van C. 1996. Phylogenetic, molecular and breeding aspects of *Azolla-Anabaena* symbiosis. *In*: M.J.Camus, M. Gibby, and R.J.Johns (eds.), Preriodology in Perspectives, Roy. Bot. Garden, Kew, pp. 611–619.

Zimmerman W.J., Lumpkin T.A. and Watanabe I. 1989. Classification of *Azolla* spp., section *Azolla*. Euphytica **43**: 223–232.

——, Watanabe I. and Lumpkin T.A. 1991a. The *Anabaena-Azolla* symbiosis: Diversity and relatedness of neotropical taxa. Plant and Soil **137**: 161–170.

——, Watanabe I., Ventura T., Payawal P. and Lumpkin T.A. 1991b. Aspects of genetic and botanical status of neotropical *Azolla* species. New Phytol. **119**: 561–566.

渡辺 巖[a]，沢本真奈美[a]，仲川亜紀子[a]，神山康夫[a]，鈴木 武[b]：**Random Amplified Polymorphic DNA法により分析したオオアカウキクサの多様性**

我が国に自生するアカウキクサ *Azolla imbricata* とオオアカウキクサ *A. japonica* のうち，オオアカウキクサについてはその実体がまだ充分に解明されていない．日本全国の27地点から採取したオオアカウキクサおよび比較として他のアカウキクサ種を用いて，これらの間の遺伝的関係を Random Amplified Polymorphic DNA（RAPD）法で分析した．その結果，オオアカウキクサは少なくとも2つのグループに分けられることが明らかとなった．そのひとつ（A グループ）には *A. filiculoides* が含まれていて，日本国内での分布は狭かった．もうひとつ（Bグループ）は広範な地域に分布していた．

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